

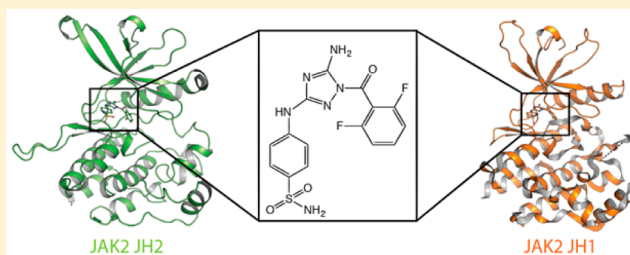
Identification and Characterization of JAK2 Pseudokinase Domain Small Molecule Binders

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Supporting Information

ABSTRACT: Janus kinases (JAKs) regulate hematopoiesis via the cytokine-mediated JAK-STAT signaling pathway. JAKs contain tandem C-terminal pseudokinase (JH2) and tyrosine kinase (JH1) domains. The JAK2 pseudokinase domain adopts a protein kinase fold and, despite its pseudokinase designation, binds ATP with micromolar affinity. Recent evidence shows that displacing ATP from the JAK2 JH2 domain alters the hyperactivation state of the oncogenic JAK2 V617F protein while sparing the wild type JAK2 protein. In this study, small molecule binders of JAK2 JH2 were identified via an *in vitro* screen. Top hits were characterized using biophysical and structural approaches. Development of pseudokinase-selective compounds may offer novel pharmacological opportunities for treating cancers driven by JAK2 V617F and other oncogenic JAK mutants.

KEYWORDS: JAK2, Pseudokinase, JH2, MPN, Small molecule



Janus kinases (JAKs) are a family of nonreceptor tyrosine kinases that signal via the conserved JAK-STAT cell signaling pathway. The four JAK family members (JAK 1–3 and TYK2) each consist of seven Janus homology (JH) domains that fold into an N-terminal FERM domain (JH7–JH5), an SH2-like domain (JH4–JH3), as well as tandem C-terminal pseudokinase (JH2) and tyrosine kinase (JH1) domains.^{1,2} Previous studies have demonstrated that the JAK pseudokinase domain is autoinhibitory of the protein's kinase domain activity, likely by direct interaction with the JH1 domain *in cis*.^{3–6} Various mutations within the JAK JH2 domain release autoinhibition, resulting in JAK hyperactivation. Specifically, the emergence of the mutant JAK2 V617F protein as the cause of the majority of myeloproliferative neoplasms (MPN) has made JAK2 an attractive target for therapeutic approaches.^{7–10} Several JAK2 inhibitors are currently in clinical trials, including momelotinib (CYT387) and pacritinib (SB1518), while the JAK1/2 inhibitor ruxolitinib has been FDA-approved for the treatment of myelofibrosis and resistant polycythemia vera.² Current JAK-targeted small molecule inhibitors used to treat MPNs are designed to target the JAK tyrosine kinase domain in an ATP-competitive manner. However, since the V617F mutation is localized within the JAK2 pseudokinase domain, these drugs do not discriminate between the wild type (WT) and mutant JAK proteins. Similarly, targeting JH1 leads to unwanted side-effects, such as neutropenia, anemia, and autoimmunity, which could be avoided or prevented should the small molecule selectively inhibit the mutant V617F protein.²

The pseudokinases of JAK1, JAK2, and TYK2 adopt a kinase fold and bind ATP with micromolar affinity.^{11–15} Recent mutagenesis studies suggest that displacing ATP from JAK2 JH2 reduces the basal and ligand-induced signaling of the full-length JAK2 V617F mutant protein while leaving the WT JAK2 unaffected. These experiments suggest that small molecule displacement of ATP from JAK2 JH2 may selectively inhibit the action of the oncogenic mutant JAK2 V617F protein.¹⁵ Thus, we sought to identify small molecules that bound selectively to the JH2 domain of the JAK2 protein and further assess to what extent these compounds bound the JH1 domain, if at all.

To this end, we performed a high-throughput fluorescence polarization (FP) screen against the JAK2 JH2 domain. The assay was designed to detect compounds that displace a fluorescently labeled ATP molecule, BODIPY-ATP, from the JH2 ATP-binding site. The JH2 protein was screened against the Selleckchem and Enzo kinase inhibitor libraries. Out of the 435 inhibitors tested, there were numerous hits that scored better than the ATP positive control (Supporting Information). Although a counter-screen against JH1 was not performed, hits were retested at multiple concentrations against both JH1 and JH2 using the fluorescence polarization assay.

The pan-CDK and Aurora A/B inhibitor JNJ-7706621¹⁶ was the top hit from the screen (Figure 1). Since the limit of

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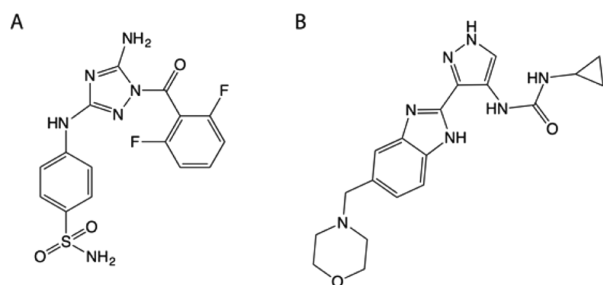


Figure 1. Chemical structures of the two top hits from the fluorescence polarization screen, (A) JNJ-7706621 and (B) AT9283.

detection for the FP assay is in the single-digit micromolar range (dictated by the K_d of ATP for the JH2 domain), the exact dissociation constant of JNJ-7706621 binding to JH2 was determined via isothermal titration calorimetry (ITC) to be 106 nM (Figure 2, Table S1), which is higher than the dissociation constant previously determined for JNJ-7706621 binding to the JAK1 JH2 domain (21 nM).¹⁷

In order to understand the molecular basis for binding, an X-ray cocrystal structure was determined of JNJ-7706621 in complex with JH2 (Figure 2). JNJ-7706621 directly interacts with the backbone of hinge residues E627 and V629 as well as the side chains of the gatekeeper residue, Q626, and conserved β 3 lysine, K581. Interestingly, in the ATP-bound structures of both the WT and V617F JH2 domains (PDB ID 4FVQ and 4FVR), there is an ordered water that hydrogen bonds to N7 of the ATP purine ring as well as Q626 and K581 and, thus, acts to further bridge ATP to the JH2 protein. This ordered water is displaced by the JNJ-7706621 carbonyl group, allowing the compound to make a direct interaction with K581 (data not shown).

We next identified whether JNJ-7706621 bound to JH1. The dissociation constant was determined via ITC to be 31 nM (Figure 3, Table S1). This value is roughly 7–8-fold tighter than the dissociation constant previously determined for JNJ-7706621 binding to the JAK2 JH1 domain (220 nM), which may be attributed to differences in assay formats.¹⁷ The affinity of JNJ-7706621 is roughly 3–4-fold tighter to JH1 over JH2. We then determined the cocrystal structure of JNJ-7706621 in

complex with JH1 in order to understand the binding mode and compare it to that of JNJ-7706621 bound to JH2 (Figure 3). The JH1 protein adopts a “DFG in” conformation and retains the canonical salt bridge between the β 3 lysine, K882, and α C-helix glutamate, E898, both of which are hallmarks of an active state protein kinase. The JNJ-7706621 compound interacts with the backbone atoms of hinge residues E930 and L932. Although JH1 lacks the analogous JH2 K581 and Q626 residues and, thus, hydrogen bonds to JNJ-7706621 (Supporting Information), the JNJ-7706621 sulfonamide amine hydrogen bonds to the L855 backbone carbonyl in the JH1 P-loop (Figure 3).

The binding mode of JNJ-7706621 to the JH1 and JH2 domains is virtually identical, again, with the main interactions being mediated by the hinge region of both proteins. However, the JNJ-7706621 compound is shifted downward in JH2 relative to compound binding in JH1. This is likely due to the fact that the analogous JH1 V863 and A880 are replaced by I559 and L579 in JH2, the latter of which both create steric bulk that forces the molecule downward (Supporting Information). Comparing the JH1 and JH2 structures to X-ray structures of other proteins crystallized with JNJ-7706621 (PDB ID 3AMA and 4QMU), the binding mode is identical (data not shown).

The known Aurora/JAK2/JAK3 inhibitor AT9283¹⁸ was also identified as a hit from the JH2 screen (Figure 1). Again, the exact dissociation constant was determined via ITC to be 11 nM and 1323 nM for JH1 and JH2, respectively (Figure 4, Table S1). The X-ray crystal structure was determined in complex with JH2 and shows a similar binding mode to that of AT9283 in complex with JH1 (PDB ID 2W1I), with the pyrazole-benzimidazole mediating hinge interactions (Figure 4). Unlike JNJ-7706621 and ATP, the AT9283 compound does not directly interact with the JH2 gatekeeper Q626. Rather, the amide carbonyl forms a water-mediated hydrogen bond to both K581 and Q626.

The binding mode of AT9283 to JH1 and JH2 is similar but with conformational differences in the cyclopropylamide and morpholino groups (Figure 4 and Supporting Information). Also, AT9283 does not enter the JH2 ATP-binding pocket to the same extent that it does in JH1. Again, this may be

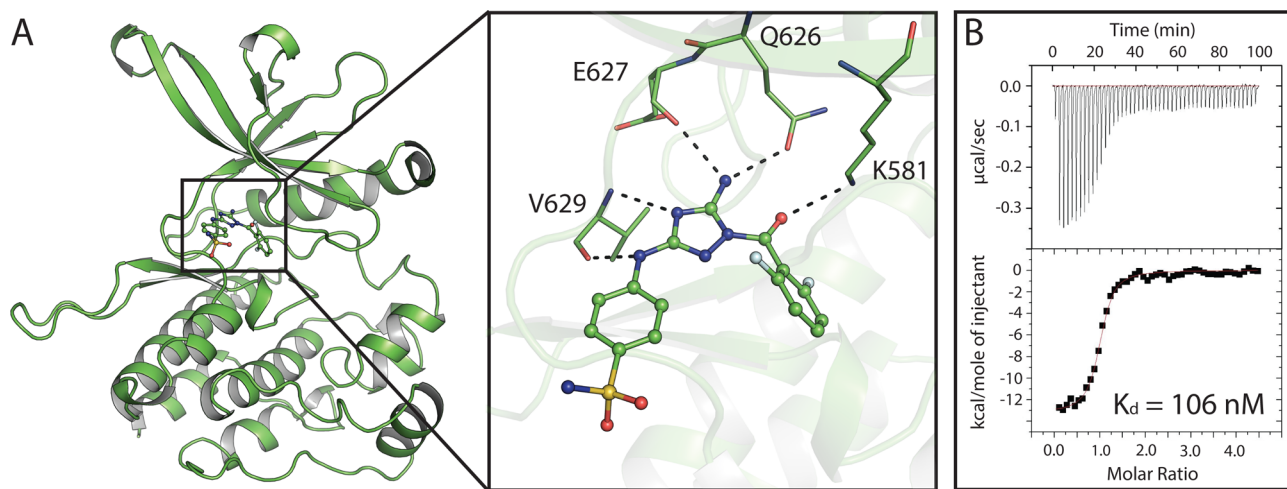


Figure 2. Characterization of JNJ-7706621 binding to the JAK2 JH2 domains. (A) Structure of the JAK2 JH2 domain in complex with JNJ-7706621. Shown is the overall JH2 domain structure and magnified view of the ATP-binding pocket. (B) Isothermal titration calorimetry analysis of JNJ-7706621 binding to JAK2 JH2.

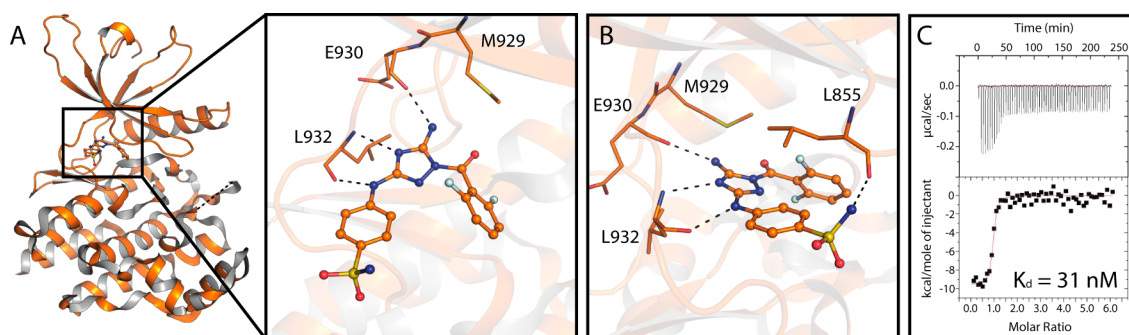


Figure 3. Characterization of JNJ-7706621 binding to the JAK2 JH1 domain. The structure contains two molecules in the asymmetric unit. Chain A is shown for parts A and B. (A) Structure of the JAK2 JH1 domain in complex with JNJ-7706621. Shown is the overall JH1 structure and magnified view of the ATP-binding pocket. (B) Different orientation of part A, showing the compound interaction with the JH1 P-loop. (C) Isothermal titration calorimetry analysis of JNJ-7706621 binding to JAK2 JH1.

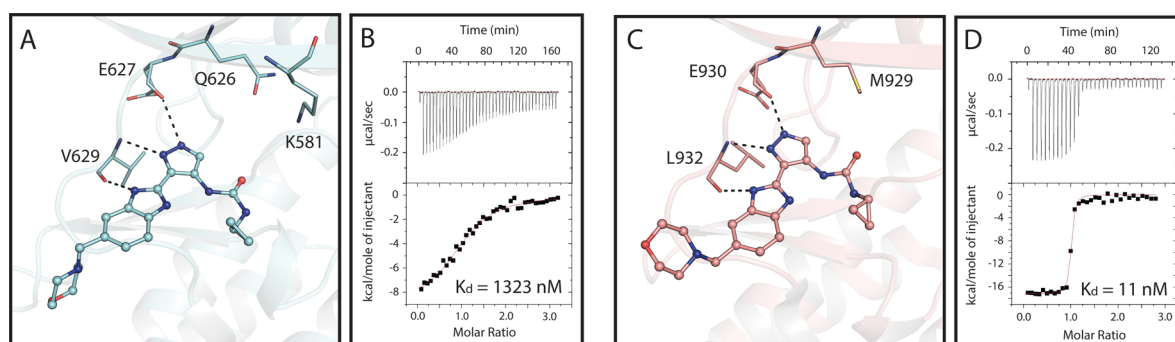


Figure 4. Characterization of AT9283 binding to the JAK2 JH1 and JH2 domains. (A) Magnified view of the ATP-binding pocket from the crystal structure of JAK2 JH2 in complex with AT9283. (B) ITC analysis of AT9283 binding to the JAK2 JH2 domain. (C) Magnified view of the ATP-binding pocket from the crystal structure of JAK2 JH1 in complex with AT9283 (PDB ID 2W11). (D) ITC analysis of AT9283 binding to the JAK2 JH1 domain.

attributed to the steric bulk of the JH2 L579 as compared to the JH1 A880, which would make the JH2 binding pocket narrower (Supporting Information).

In closing, we have identified small molecules that bind to the pseudokinase domain of JAK2. The top hit, JNJ-7706621, binds to both JH1 and JH2 with nanomolar affinity. The current compounds primarily bind to the JH2 hinge region (Figures 2–4, Supporting Information). Since residues F594 and F595 in the JH2 α C-helix are essential for the hyperactivation of the V617F mutant,^{15,19} medicinal chemistry efforts will focus on extending compounds into the JH2 pocket, likely toward the α C-helix (Supporting Information). Once pseudokinase-selective small molecules are obtained, we will further determine whether such compounds selectively inhibit the constitutive activation of the oncogenic mutant JAK2 V617F protein.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.7b00153.

Detailed procedures, supporting figures, showing sequence alignment, electron density maps, and chemical structures, and tables, showing thermodynamic parameters, and data collection and refinement statistics (PDF) Hit list from the fluorescence polarization screen (XLSX)

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Notes

The authors declare no competing financial interest.

X-ray crystal structures were deposited in the RCSB Protein Data Bank with the following codes: JAK2 JH1/JNJ-7706621 (PDB ID 5USY); JAK2 JH2/JNJ-7706621 (PDB ID 5USZ); JAK2 JH2/AT9283 (PDB ID 5UT0); JAK2 JH2/BI-D1870 (PDB ID 5UT1); JAK2 JH2/PRT062607 (PDB ID 5UT2); JAK2 JH2/IKK-2 Inhibitor VI (PDB ID 5UT3).

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■ ABBREVIATIONS

JAK, Janus kinase; JH, Janus homology; JH1, JAK kinase domain; JH2, JAK2 pseudokinase domain; FP, fluorescence polarization; K_d , dissociation constant; ITC, isothermal titration calorimetry

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